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Effects of some environmental factors on growth and nitrate reductase of corn seedlings.

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EFFECTS OF SOME ENVIRONMENTAL FACTORS ON GROWTH AND NITRATE REDUCTASE OF CORN SEEDLINGS

A Dissertation Presented

by

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D E D I C A T I O N

To my wife Carmelita

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I wish to extend my heartfelt gratitude to Dr. John R. Havis, chairman of the Committee, and Dr. Herbert V. Marsh, Jr. and Dr. William J. Bramlage, members of the Committee for their continued guidance and direction during the course of this research work and for their thorough and critical review of this dissertation.

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GROWTH AND NITRATE REDUCTASE ACTIVITY IN WATER-STRESSED CORN SEEDLINGS

APPENDIX

GROWTH AND NITRATE REDUCTASE ACTIVITY IN WATER-STRESSED CORN SEEDLINGS[1]

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[1]This study is part of a dissertation submitted by the senior author in partial fulfillment of the requirement for a Ph.D. in Plant and Soil Sciences at the University of Massachusetts.

ABSTRACT

Leaf elongation and nitrate reductase activity in water-stressed corn (Zea mays L., cv. WF9 x M14, Crow's hybrid corn, Milford, Ill.) seedlings were determined. Leaf elongation stopped with 6.5% or higher water deficit. Water stress reduced nitrate reductase activity only at values appreciably higher than those required to stop leaf elongation. Water deficits of 7 to 17% only slightly reduced nitrate reductase activity, but higher water deficits reduced the activity of the enzyme significantly.

There appeared to be an inhibitor present in the leaf tips of the corn seedlings which inhibited nitrate reductase. An acetone fraction prepared from a tip extract lowered the apparent level of exogenously added NO_2^-. This factor was found to be heat labile, was sensitive to temperature, $HgCl_2$, and iodoacetate, and could be removed by Polyclar AT. There was also an inhibitor of nitrate reductase in extracts of the leaf tip which was not removed by Polyclar AT treatment. The presence of this inhibitor was not related to water stress conditions.

INTRODUCTION

Water stress reduces the growth rate of plants (31). This is at least partially due to a reduction in photosynthesis (5, 9, 29, 30). However, other stress-induced metabolic changes are known to occur, and their relationship to growth reduction and growth resumption on recovery from water stress are unclear. Crafts (7) reported that important metabolic reactions and overall physiological functions change in most cells and tissues long before the air-dried state is reached. Amino acid as well as protein synthesis decreased when plants were subjected to only mild moisture stress conditions (4, 18, 27, 34). These dramatic changes can cause shifts in overall metabolism in the plant which may lead to changes in the capabilities of various enzymes in performing their physiological functions. Because of the effect of water stress on protein synthesis one should study a system where changes occur within a short period. Nitrate reductase has a short half-life and was therefore selected for this study. This paper is concerned with the effect of water stress on the activity of the enzyme nitrate reductase and on the the growth of corn seedlings.

MATERIALS AND METHODS

Plant Material. Seeds of corn (Zea mays L., cv. WF9 x M14, Crow's hybrid corn, Milford, Ill.) were soaked in aerated tap water for 24 hr and sown in vermiculite or soil. The plants grown in soil were watered once a week with full strength Hoagland (14) solution, while those germinated in vermiculite were supplied with the same nutrient solution at least twice a week. Seedlings germinated invermiculite were removed when 3 to 4 cm in height and fitted loosely into holes made on circular styrofoam boards. These boards, each holding 16 seedlings, were placed on 1 liter black plastic pots filled with 1/3 strength aerated Hoagland solution which was changed twice a week. These seedlings were grown in the greenhouse to their early 5-leaf stage before being transferred to a growth chamber (3,300 ft-c, 25°C, 40-50% R.H.) where stress treatments were applied. The seedlings were held under a 12-hr light and 12-hr dark cycle for 2 days after which the appropriate light regimes were initiated.

Stress Treatments. Stress treatments were started 2 days after placing the seedlings in the growth chamber. Stress was initiated by withholding water from soil-grown seedlings; for those in nutrient solution, stress was initiated by transfer of the seedlings into a solution containing the full complement of essential elements but

with 500 ml of 45% (w/v) polyethylene glycol-6000 (PEG-6000) diluted to 1 liter with Hoagland solution. Water deficit was measured using the method of Weatherly (32) as modified by Bardzik et al. (3) and was expressed as the ratio of water taken up by leaf tissue when floated on water divided by the final water content, multiplied by 100. Water potential was measured with the pressure bomb as described by DeRoo (8). The middle leaf (third leaf) was used in determining the water status of the plants. Water deficit and water potential relationships in some plants used in this study are shown in Figure 1.

Growth Measurements. The elongation of the leaves of corn plants grown in vermiculite was measured during the development of water stress under a continuous light regime. The length of the fourth leaf was measured from the base of the blade of the third leaf to the tip of the fourth leaf.

Enzyme Preparation. The middle and tip portions of leaf blades were weighed separately, chilled, and used as sources of nitrate reductase. Three g of leaf tissue were sliced and ground in a VirTis homogenizer at high speed for 1 min. The grinding medium contained 0.1M TRIS (tris-hydroxymethyl, aminomethane), pH 7.5, 0.01M cysteine (L-cysteine hydrochloride·H_2O) and 0.0003M EDTA (ethylene-dinitrilotetraacetic acid). Four ml of cold (2°C) grinding

medium was used for each g of tissue. In some experiments, Polyclar AT was added to the grinding medium (1 g/g tissue) after it had been thoroughly washed and tested to be free of nitrite. The homogenate was squeezed through a double layer of cheesecloth and centrifuged at 22,000g for 15 min. The supernatant was used for enzyme assay either directly ("crude fraction") or after acetone precipitation. The "acetone fraction" was prepared by adding with stirring 1.5 volumes (v/v) of cold (-10°C) acetone to the supernatant. The solution was held for 20 min at -10°C and then centrifuged for 10 min at 12,000g to sediment the flocculated material. The precipitate was washed with distilled water at least 3 times and resuspended in 0.1M phosphate (K^+) buffer (pH 7.6) which is equal in volume as the supernatant used in acetone precipitation.

Nitrate Reductase Assay and Protein Analysis.

Nitrate reductase activity was estimated using a slightly modified method of Hageman and Flesher (11). The assay medium consisted of 1.1 ml of 0.1M phosphate buffer, pH 7.6, 0.2 ml of 0.1M KNO_3, 0.5 ml of 4.53×10^{-4}M NADH and 0.2 ml of the enzyme preparation. Distilled water was substituted for NADH in the controls. The reaction was initiated by adding NADH, and the mixture was incubated at 30°C for 10 to 30 min. The reaction was stopped by adding 1 ml of 1% (w/v) sulfanilamide in 1.5N HCl. The color was developed by adding 1 ml of 1% (w/v) N-(1-naphthyl)-ethylene diamine

dihydrochloride solution and the absorbancy was determined after 5 min against a blank in Beckman DU-2 spectrophotometer at 540 nm. The enzyme activity was expressed as either umoles of NO_2^- formed/mg protein·min or umoles of NO_2^- formed/ g fresh weight·hr. Protein was determined by the biuret method using bovine serum albumin as standard (20).

RESULTS

Leaf elongation of corn seedlings was greatly reduced by water stress (Table 1). A water deficit of only 3.5% sifnificantly reduced leaf elongation, and elongation of the leaf ceased with 6.5% or higher water deficit. No visible symptoms of wilting were observed in leaves of corn seedlings with a water deficit of 6.5%. Growth rate declined with time for the control, presumably due to aging of the leaves.

Nitrate reductase activity was reduced by water stress in plants held under continuous light, whether the stress was achieved by continuous drying of the soil or by use of an osmoticum (Table 2). Water stress values of 36.5% and 24.5% in soil and PEG-6000, respectively, were obtained 72 hr after the stress was initiated. The water stress values here were much higher than the amount of stress required to stop growth (Table 1). In a subsequent experiment (Table 3), water stresses of 7 to 17% were measured and nitrate reductase activity was only slightly reduced, if at all. It made little difference whether the plants were stressed in soil or in PEG-6000, or whether measurements were made during the light or dark cycles, although nitrate reductase activities were reduced by darkness.

It was observed that crude extracts prepared from the tips of leaves contained consistently less nitrate reductase

activity than extracts from the middle portion of the same leaves (Table 4). This tip activity was reduced much more than the mid-portion activity by stress. When extracts of the leaf tip were mixed with extracts of the middle portion, a marked reduction, rather than an average enzyme activity, was measured in the mixture (Table 5). This observation suggested the presence of an inhibitory substance in the tip of the leaves. Acetone fractions of thetip extract had greater inhibitory power than the crude preparation (Table 5). Because of this observation, only acetone fractions were used as sources of inhibitors in all succeeding experiments. A batch of test plants was grown in the greenhouse, extracted for nitrate reductase and used as a homogeneous source of enzyme to test for the presence of inhibitory factors in the acetone fraction of the tip preparation.

Table 6 compares the inhibitory activity of the acetone-precipitated tip extracts from the control and the stressed plants. The data show that the acetone fraction of the tip extract inhibited the activity of the test plant enzyme whether the source of the inhibitor was the stressed or the control plants. In no case did the tip inhibitor result in complete inhibition of the nitrate reductase activity of the test plant. However, it appeared that greater inhibition occurred using tips from stressed than from control plants.

It was thought that greater inhibition might result

if the tip extract were pre-incubated with the enzyme for a longer period before assay. However, it was found (Table 7) that when the tip extract and the extract from the test plants were pre-incubated for 20 min at 30°C before assay, an apparent enhancement rather than a reduction of activity occurred. This was true in both the control and the stressed plants. These unexpected findings were found to be at least in part due to an increase in enzyme activity in the test plants with preincubation. For example, when test plant extract was pre-incubated for 20 min at 30°C activity was found to rise from 0.91 ± 0.10 umoles NO_2^-/g·hr (without preincubation) to 1.70 ± 0.03 umoles NO_2^-/g·hr (with preincubation).

Another possibility which may have contributed to the apparent increase in nitrate reductase activity upon preincubation was that the NO_2^- being produced in the standard nitrate reductase assay was also being partly dissipated, and that this partial loss of nitrite was decreased during or as a result of the preincubation. To test this possibility experiments were conducted in which a constant amount of NO_2^- was added to the test plant extract in the presence or absence of tip extracts and the amount of NO_2^- remaining after varying treatments was determined by the standard sulfanilamide-ethylene diamine dihydrochloride assay. Table 8 shows that loss of total nitrite was less with longer preincubation periods although some loss

occurred in a 10 min reaction period even after a 60 min preincubation.

Previous experiment (Table 5) showed that the tips of corn leaves contained a factor (or factors) that exerts an inhibitory effect on the activity of nitrate reductase. Moreover, this factor(s) caused the loss of NO_2^- when exogenous NO_2^- was added to test plant extracts along with nitrate reductase cofactors (Table 8). To determine if the factor were heat-lable, some extracts were boiled and NO_2^- loss was determined. Boiling the tip extract resulted in the complete removal of the factor responsible for the apparent loss of exogenously added NO_2^-, whereas in the unboiled sample NO_2^- decreased substantially in 10 min (Table 9). It was also found that the apparent loss of added NO_2^- was influenced by the reaction temperature, with loss being greater at higher temperatures (Table 10). This temperature effect was absent, however, when the boiled extract was used. Again, however, boiling did not completely prevent the apparent loss of NO_2^-. The amount of the tip extract also influenced the apparent loss of NO_2^- (Table 11). Higher amounts of extract resulted in greater loss of total NO_2^-in unboiled preparations. Some loss also occurred in the boiled sample, and this loss increased with increased amount of extract.

Two metabolic poisons were used in an effort to further characterize the nature of the inhibitor. A metallic poison, $HgCl_2$ (1×10^{-3}M), and an alkylating agent, iodoacetate (200 ppm), were used; these concentrations completely inhibited nitrate reductase activity in preliminary tests. The data in Table 12 suggest that these poisons decreased the ability of the tip extract to remove NO_2^-, although neither substance was as effective as boiling.

To minimize any inhibitory effects of phenolic compounds which may be released during the grinding process, Polyclar AT, an insoluble polyvinylpyrrolidone, was used in the homogenizing mixture. Nitrate loss, as shown by the NO_2^- reaction test, no longer occurred when NO_2^- was added to the boiled or unboiled acetone fractions of the tip extract (Table 13). However, nitrate reductase activity of mid-portion or crude extract was still reduced by water stress (Table 14). The overall activity of the enzyme was consitently higher in Polyclar AT-treated fractions than in enzyme fractions homogenized in absence of Polyclar AT. For example, the control values for nitrate reductase in plants that were homogenized without Polyclar AT ranged from 0.86 to 1.20 umoles/mg protein·min while values of 3.5 to 4.56 umoles were observed in plants with Polyclar AT (cf Tables 2, 3, 4 and 14). Inhibition of nitrate

reductase activity by acetone fractions from the tip was still observed, although this was no longer sensitive to boiling; in fact, it appeared to be enhanced by boiling (Table 15).

DISCUSSION

Water stress reduced nitrate reductase activity in corn seedlings. The same result was also reported by Bardzik *et al.* (3), but they did not demonstrate the effect of increased water deficit on growth. Our present work shows that although 6.5% water deficit stopped leaf elongation of corn seedlings, a much greater amount of water stress was required to significantly reduce nitrate reductase activity. It seems that growth processes are much more sensitive to water stress than factors regulating nitrate reductase activity. Other workers have also shown that extension growth is extremely sensitive to changes in turgor pressure. Elongation of *Nitella* cells, for example, was reduced by decreases in turgor pressure of fractions of a bar and stopped by a decrease of 3 bars (10). Elongation of maize leaves was also shown by Hsiao *et al.* (16) to be similarly affected by slight decreases in tissue water potential. In another work, Hsiao (15) indicated that reduction inshoot elongation of etiolated corn seedlings preceded reduction in the number of polysomes at the onset of water stress. Although nitrate reductase has been reported to be extremely sensitive to slight depletion of moisture (3, 17, 24, 33), the present evidence showed that elongation of the leaves in corn seedlings seems to be much more sensitive.

If one assumes that the activity of nitrate reductase is a good index of the status of cytoplasmic enzymes which are rapidly turning over, then these results would suggest that the inhibition in growth of the seedlings as the tissue becomes desiccated can not be accounted for on the basis of the loss of activity of enzymes with half-lives of 4 hr or more. This, of course, does not exclude a possible effect of the stress on short-lived enzymes such as the so-called "growth-limiting proteins" described by Cleland (6), with an apparent life of 20 min.

Many plants are relatively rich in phenolic compounds which have often caused problems in studying enzymes isolated from plant sources (1, 12, 13). Although these phenolics are thought to combine with the proteins to form enzymatically inactive complexes, it is also conceivable that these compounds might interact with NO_2^-, or the reagents added to develop the nitrite-sulfanilamide pigment used to estimate the amount of NO_2^- present, and thereby cause an apparent reduction in the amount of NO_2^-. In recent years compounds which form insoluble complexes with phenolic compounds have been used to remove the phenolics from enzyme preparations. One such phenol-complexing reagent is Polyclar AT, an insoluble polyvinylpyrrolidone, which has been used by a number of workers (2, 21). The results presented in Table 13 show that added NO_2^- was completely stable to boiling in extracts prepared in the

presence of Polyclar AT. There was no substantial difference between boiled and unboiled extracts or extracts prepared from stressed or non-stressed plants. Apparently then, the factor responsible for the apparent loss of NO_2^- (Tables 9-12) was completely removed by Polyclar AT.

Because of the effect of Polyclar AT on the stability of NO_2^- in the reaction mixture, the effects of water stress on nitrate reductase activity were re-examined suing extracts prepared with Polyclar AT (Table 15). Reductase activities 3-to 4-fold greater than had previously been found (cf Tables 2-3-4) were obtained by grinding with Polyclar AT. Reductase activity was still depressed in severely stressed tissue (Table 14); however, it was by no means totally inactivated. It is also interesting that the extracts prepared from the tips also contained strong nitrate reductase activity and that acetone precipitation did not lead to a complete loss of this activity.

Many reducing agents have been shown to affect nitrate reductase. Vennesland and Jetschmann (28) reported that NADH can lead to the loss of the activity of the enzyme. NADH was also reported by Stulen (26) to interfere with the estimation of the NO_2^- produced by nitrate reductase. It has been customary to add a reducing agent like ascorbate during extraction of enzymes, but this compound has also been reported by Mason (23) to activate the ortho-hydroxylation of monophenols by phenol oxidase and can therefore be

harmful. Ammonia, according to Losada *et al*. (22), can cause the inactivation of nitrate reductase in *Chlamydomonas* by indirectly reducing the enzyme. It is possible that in our study the majority of the apparent inhibitors present in the acetone fraction of the tip extract were in fact reducing agents and may even be reduced forms of phenols and were acting in the same manner as the above-mentioned reducing agents. These phenolics and reducing agents were probably removed when the enzyme was prepared with Polyclar AT. Perhaps some of the reducing agents were actually oxidized upon standing for 20 min at 30°C when the tip inhibitor-test plant mixture was preincubated, thus preventing them from reducing the enzyme.

The question persists whether or not the apparent inhibition of nitrate reductase activity caused by the acetone-precipitated tip extracts can be explained by the materials removed by Polyclar AT. The data presented in Table 15 show that some inhibitory factor still remained in extracts prepared with Polyclar AT. Interestingly, boiling these extracts appeared to increase the effectiveness of these factors. It is not clear at this point what is responsible for this apparent inhibition. There is, however, no clear relationship between water stress and this inhibition.

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FIGURE 1

Relationship Between Percentage Water Deficit and Leaf Water Potential in Corn Seedlings.

The leaf water potential of corn seedlings was measured each time the percentage water deficit was determined. The procedures used are stated in the Materials and Methods. The circles are actual values measured for water deficit and water potential. The square are values of the same parameters representing the best fit line determined through a computerized program Least Square Fit (Appendix Tables 1 and 2).

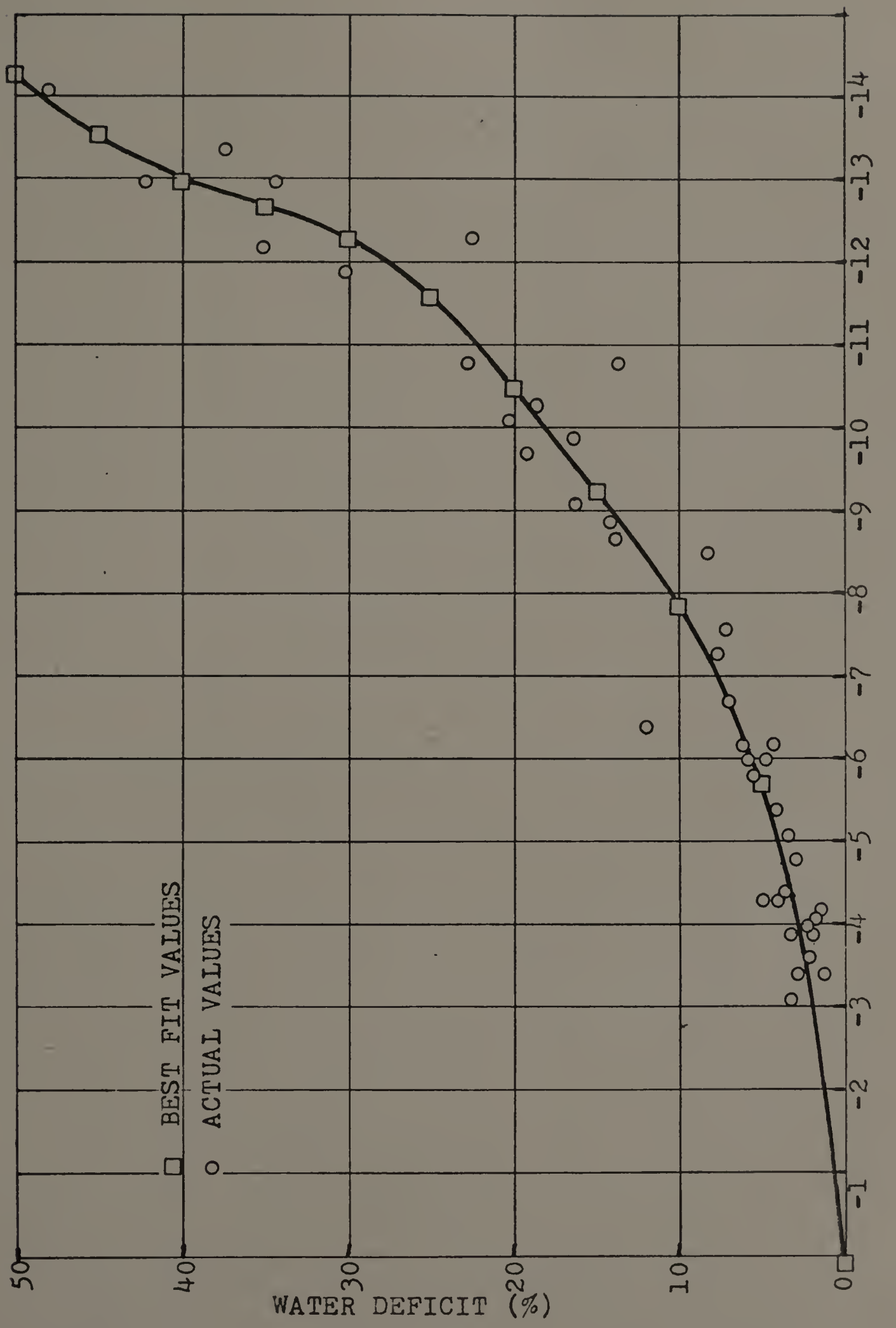
LEAF WATER POTENTIAL (BARS)
-1
-2
-3
-4
-5
-6
-7
-8
-9
-10
-11
-12
-13
-14
WATER DEFICIT (%)
0
10
20
30
40
50
BEST FIT VALUES
ACTUAL VALUES

FIGURE 1

Table 1. Elongation of corn leaves subjected to water stress under continuous light.

Stress was applied by withholding water from plants growing in vermiculite. Water deficit and leaf elongation were determined every 12 hr after the initiation of stress. The data are averages of 3 replications.

Hours of Stress at 25°C	Increase in Length	
	Control	Stressed
	mm/hr	
0-12	1.77 ± 0.05 (2.1)[1]	1.69 ± 0.03 (2.8)
12-24	1.68 ± 0.05 (1.8)	0.94 ± 0.37 (3.5)
24-36	1.53 ± 0.02 (2.1)	0.89 ± 0.02 (5.2)
36-48	1.22 ± 0.06 (0.5)	0.01 ± 0.00 (6.5)
48-60	0.94 ± 0.03 (1.4)	0.00 ± 0.00 (29.7)

[1]Numbers in parentheses are water stress measurements in % water deficit.

Table 2. Nitrate reductase activity of the control and water-stressed corn seedling leaves under continuous light.

Determinations of water deficit and nitrate reductase activity were made 72 hr after stress was initiated. The data are averages of 3 replications.

Medium	Control	Stressed
	umoles NO_2^-/mg protein·min	
Soil	1.19 ± 0.02 (3.9)[1]	0.65 ± 0.02 (36.5)
PEG-6000	0.86 ± 0.04 (2.2)	0.29 ± 0.03 (24.5)

[1]Numbers in parentheses are water stress measurements in % water deficit.

Table 3. Nitrate reductase activity of leaves from control and stressed corn seedlings under a 12-hr light and 12-hr dark regime.

Determinations of water deficit and nitrate reductase activity were made 72 and 84 hr after water stress was initiated for the light and dark measurements, respectively. Measurements were made at the end of the indicated portion of the light regime. The data are averages of 3 replications.

Light Regime	Control	Stressed
	umoles NO_2^-/mg protein·min	
Soil-Grown Plants		
Light	1.20 ± 0.04 (2.3)[1]	1.29 ± 0.06 (7.2)
Dark	0.82 ± 0.05 (1.1)	0.63 ± 0.04 (7.0)
PEG-6000 Plants		
Light	0.92 ± 0.03 (3.6)	0.84 ± 0.03 (17.3)
Dark	0.85 ± 0.01 (1.1)	0.76 ± 0.02 (13.1)

[1]Numbers in parentheses are water stress measurements in % water deficit.

Table 4. Nitrate reductase activity of the tip and middle portions of the leaves of the control and stressed corn seedlings.

Measurements of water deficit and nitrate reductase activity were made 72 hr after stress was initiated. The data are averages of 3 replications.

Leaf Portion	Control	Stressed
	umoles NO_2^-/mg protein·min	
Soil-Grown Plants		
Middle	1.20 ± 0.04 (2.3)[1]	1.29 ± 0.06 (7.2)
Tip	0.94 ± 0.07 (2.3)	0.62 ± 0.32 (7.2)
PEG-6000 Plants		
Middle	0.92 ± 0.03 (3.6)	0.84 ± 0.03 (17.3)
Tip	0.81 ± 0.02 (3.6)	0.33 ± 0.08 (17.3)

[1]Numbers in parentheses are water stress measurements in % water deficit.

Table 5. Nitrate reductase activities of crude and acetone fractions from leaves of control and stressed corn seedlings.

Measurements of water deficits and nitrate reductase activity were made after 36 hr stress in PEG-6000. The data are averages of 3 replications.

Enzyme Source	Crude Fraction	Acetone Fraction
	umoles NO_2^-/mg protein.min	
Control (Mid-portion)	0.85 ± 0.16 (1.1)[1]	1.12 ± 0.12 (1.1)
Control (Tip)	0.28 ± 0.01	0.04 ± 0.00
Stressed (Mid-portion)	0.76 ± 0.10 (13.1)	1.02 ± 0.02 (13.1)
Stressed (Tip)	0.42 ± 0.06	0.00 ± 0.00
Control (Mid-portion) + Control (Tip)	0.40 ± 0.04[2]	0.37 ± 0.07[3]
Control (Mid-portion) + Stressed (Tip)	0.40 ± 0.02[2]	0.06 ± 0.02[3]

[1]Numbers in parentheses are water stress measurements in % water deficit.

[2]Mixtures include 0.2 ml crude fraction of control (mid-portion) and 0.2 ml crude fraction of designated tip.

[3]Mixtures include 0.2 ml crude fraction of control (mid-portion) and 0.2 ml acetone fraction of designated tip.

Table 6. Inhibition of nitrate reductase activity of crude fraction of test plant by acetone fractions from leaf tips of control and water-stressed corn seedlings.

Water deficit and nitrate reductase activity were measured 48 hr after stress was initiated. Test plants were not stressed and were used only as source of enzyme for testing the tip inhibitors. The data are averages of 3 replications.

Source of Acetone Fractions	Test Plant	Tip	Test Plant Plus Tip[1]
		umoles NO_2^-/gfw·hr	
Soil-Grown Plants			
Control Tips (1.6)[2]	3.23 ± 0.03	0.66 ± 0.05	1.36 ± 0.05
Stressed Tips (12.2)	3.23 ± 0.03	0.00 ± 0.00	0.81 ± 0.02
PEG-6000 Plants			
Control Tips (3.5)	5.86 ± 0.12	0.00 ± 0.00	1.38 ± 0.08
Stressed Tips (8.4)	5.86 ± 0.12	0.00 ± 0.00	1.22 ± 0.10

[1]Mixtures include 0.2 ml crude fraction of the test plant (mid-portion) and 0.2 ml acetone fraction of designated tip.

[2]Numbers in parentheses are water stress measurements in % water deficit.

Table 7. Nitrate reductase activity in control and water-stressed corn seedling leaves 20 min after pre-incubation of mixtures of the test plant enzyme and the acetone fraction of the tip enzyme at 30°C.

Water deficit and nitrate reductase activity were measured after 48 hr stress in PEG-6000. The data are averages of 3 replications.

Source of Acetone Fraction	Test Plant	Test Plant Plus Tip[1]	
		No Preincubation	Preincubation
		umoles NO_2^-/gfw·hr	
Control (3.5)[2]	5.86 ± 0.12	1.27 ± 0.12	7.12 ± 0.22
Stressed (8.4)	5.86 ± 0.12	1.18 ± 0.02	6.54 ± 0.23

[1]Mixtures include 0.2 ml crude fraction of the test plant (mid-portion) and 0.2 ml acetone fraction of designated tip.

[2]Numbers in parentheses are water stress measurements in % water deficit.

Table 8. Effect of preincubation of a nitrate reductase preparation for different intervals on the subsequent apparent loss of exogenously added nitrite.

Crude extracts from test plants were preincubated for varying time periods at 30°C, after which a constant amount of NO_2^- was added along with the cofactors for nitrate reductase assay. The total NO_2^- in the absence of the extract was 32.2 ± 0.6 mumoles. The data are averages of 3 replications.

Preincubation Time	Initial	After 10 min Reaction Period
min	mumoles NO_2^-	
20	31.00 ± 0.00	15.33 ± 0.62
30	31.00 ± 0.20	22.33 ± 0.24
40	31.00 ± 0.40	23.73 ± 1.28
60	30.75 ± 8.75	25.33 ± 1.02

Table 9. Apparent change in NO_2^- content in boiled and unboiled acetone fractions from tips of control and stressed corn seedlings.

Water deficits and nitrite were measured after 48 hr stress in soil. Water deficits of the control and stressed plants were 4.6% and 48.8%, respectively. A constant amount of NO_2^- was added along with the cofactors for nitrate reductase assay to tip extracts. Total NO_2^- in absence of tip extract was 27.16 ± 1.10 mumoles. The data are averages of 3 replications.

Reaction Period	Boiled		Unboiled	
	Control	Stressed	Control	Stressed
	mumoles NO_2^-			
Initial	24.40 ± 0.29	26.30 ± 0.28	17.03 ± 0.20	16.07 ± 0.12
After 10 Min	27.83 ± 0.47	29.03 ± 1.37	11.50 ± 0.70	10.50 ± 0.41

Table 10. Effect of reaction temp on NO_2^- loss from acetone fractions of non-stressed corn leaf tips.

Procedure as in Table 9. Total NO_2^- in absence of extract was 27.60 ± 0.29. The data are averages of 3 replications.

Reaction Temp	Amount of NO_2^- Remaining after 10 Min Reaction	
	Unboiled	Boiled
°C	mumoles	
0	14.33 ± 0.62	18.70 ± 0.62
20	5.16 ± 0.23	21.60 ± 0.82
30	2.88 ± 0.23	21.70 ± 0.70
40	2.66 ± 0.24	20.06 ± 1.02

Table 11. Effect of increasing amounts of acetone fraction from non-stressed corn leaf tips on the loss of NO_2^-

A constant amount of NO_2^- was added to varying amounts of boiled and unboiled acetone fractions. Procedure as in Table 9. Total NO_2^- in absence of extract was 27.56 ± 0.38 mumoles. The data are averages of 3 replications.

Amount of Extract	Amount of NO_2^- Remaining after 10 Min at 30°C	
	Unboiled	Boiled
ml	mumoles	
0.1	18.23 ± 0.75	26.80 ± 0.24
0.2	8.23 ± 0.20	25.56 ± 0.66
0.3	5.20 ± 0.14	22.76 ± 0.38

Table 12. Effects of boiling and metabolic poisons on loss of NO_2^- from acetone fractions of unstressed corn leaf tips.

The metabolic poisons were mixed with the tip fraction 10 min before assay. Procedure as in Table 9. Total NO_2^- in absence of extract was 27.47 ± 0.69 mumoles. The data are averages of 3 replications.

Treatment	Amount of NO_2^- Remaining after 10 Min Reaction
	mumoles
Boiled Fraction	27.20 ± 0.22
Unboiled Fraction	13.60 ± 0.43
Unboiled Fraction + $HgCl_2$ [1]	17.73 ± 0.53
Unboiled Fraction + Iodoacetate [2]	16.86 ± 0.35

[1] $HgCl_2$ concentration = 1×10^{-3}M

[2] Iodoacetate concentration = 200 ppm

Table 13. Loss of NO_2^- from acetone fraction from unstressed corn leaf tips homogenized with Polyclar AT.

Procedure as in Table 9. Total NO_2^- in absence of extract was 27.10 ± 0.14 mumoles. The data are averages of 3 replications.

Water Deficit	Unboiled Tip	Boiled Tip
%	mumoles NO_2^-	
2.4	27.80 ± 0.63	28.03 ± 1.39
3.0	26.20 ± 0.99	27.43 ± 0.82
19.2	29.96 ± 0.87	31.00 ± 0.82

Table 14. Effect of water deficit on nitrate reductase activity of extracts from middle and tip portions of corn leaves homogenized with Polyclar AT.

The middle and tip portions of the leaf were ground separately. Polyclar AT was added to the grinding medium at the rate of 1g/ g leaf tissue. The data are averages of 3 replications.

Water Deficit	Middle Portion	Tip Portion	
		Crude Fraction	Acetone Fraction
%	umoles NO_2^-/mg protein·min		
2.4	4.56 ± 0.62	2.27 ± 0.08	0.57 ± 0.07
3.0	3.56 ± 0.14	2.05 ± 0.10	1.04 ± 0.02
19.25	2.38 ± 0.17	1.69 ± 0.08	0.99 ± 0.02

Table 15. Inhibition of test plant nitrate reductase by acetone fractions of leaf tips from corn seedlings under different water stress.

Both test plants and stressed plants were homogenized with Polyclar AT (1g/g leaf tissue). Mixtures consisted of 0.2 ml test plant extract and 0.2 ml tip extract. The activity of test plant extract alone was 1.12 ± 0.04 umoles NO_2^-/mg protein·min. The data are averages of 3 replications.

Water Deficit	Test Plant Plus Unboiled Tip	Test Plant Plus Boiled Tip
%	umoles NO_2^-/mg protein·min	
2.4	0.69 ± 0.06	0.59 ± 0.07
3.0	0.90 ± 0.05	0.49 ± 0.05
19.2	0.91 ± 0.04	0.60 ± 0.08

GROWTH AND NITRATE REDUCTASE ACTIVITY OF CORN SEEDLINGS DURING RECOVERY FROM WATER STRESS[1]

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[1]This study is part of a dissertation submitted by the senior author in partial fulfillment of the requirement for a Ph.D. in Plant and Soil Sciences at the University of Massachusetts.

ABSTRACT

Elongation of intact leaves and activity of nitrate reductase in corn (Zea mays L., cv. WF9 x M14, Crow's hybrid corn, Milford, Ill.) seedlings were studied during recovery from water stress. Plants which had 36.2% water deficit and were not growing after 48 hr of stress showed an apparent surge in growth 4 hr after they were rewatered and their water status returned to the control level. This surge in growth was followed by a slower rate of elongation in the next 8 hr of recovery. After 12 hr, the stressed plants grew at the same rate as the control.

Nitrate reductase activity of the stressed plants increased 18 to 24 hr after rewatering in continuous light. In continuous darkness, nitrate reductase activity of both the control and stressed plants decreased over the 48 hr recovery period.

Application of cycloheximide to plants during recovery reduced but did not eliminate the increase in enzyme activity in light. Thus, both synthesis of new enzyme and reactivation of pre-existing enzyme appear to occur during recovery. However, the reactivated portion of the nitrate reductase enzyme did not persist for long, because activity declined sharply after 24 hr.

Addition of nitrate during recovery induced enzyme activity in plants whether in light or darkness. In darkness induction was equal in both control and stressed plants but in light, stressed plants developed twice as much activity as the controls.

INTRODUCTION

Our previous paper (8) showed that both growth and nitrate reductase activity were reduced by water stress in young corn seedlings. A similar effect of water stress on growth was reported by Hsiao *et al.* (18) and Boyer (6). While a number of workers have investigated recovery of growth of plants (12, 13), including corn (1, 18), after rewatering, there are few reports on the recovery of enzyme activity upon release of water stress. Bardzik *et al.* (3) found some recovery of nitrate reductase activity after corn plants had recovered from water stress, but characterization of the recovered enzyme activity and its relation to recovery in growth were not reported.

This study was undertaken to examine the nature of and relationships between the recovery of growth and of nitrate reductase activity upon release of water stress in corn seedlings.

MATERIALS AND METHODS

Plant Materials. Seeds of corn (Zea mays, L., cv. WF9 x M14, Crow's hybrid corn, Milford, Ill.) were soaked in aerated tap water for 24 hr, sown in vermiculite, and supplied with full strength Hoagland (16) solution at least twice a week. Seedlings for solution culture were removed from vermiculite at 3 to 4 cm height and fitted loosely into holes made on circular styrofoam boards. These boards, each holding 16 seedlings, were placed on 1 liter black plastic pots filled with 1/3 strength, aerated Hoagland solution which was changed twice a week. These seedlings were grown in the greenhouse to the early 5-leaf stage before being transferred to a growth chamber at 25°C, illuminated to 3,300 ft-c. The light periods were varied according to the needs of the experiment. The relative humidity in the growth chamber was adjusted to 40-50% when the plants were stressed and to 75% when the plants were allowed to recover from water stress.

Stress and Recovery Treatments. Water stress was induced (a) by withholding water from seedlings grown in vermiculite, or (b) by placing seedlings growing in nutrient solution into 500 ml of 45% (w/v) polyethylene glycol-6000 (PEG-6000) diluted to 1 liter with Hoagland solution. Water deficit was measured using the method of Weatherley (32) as modified by Bardzik (3) and was

expressed as the ratio of water taken up when the leaf tissue was floated on water divided by the final water content of the tissue, multiplied by 100. Stressed plants grown in vermiculite were rewatered with tap water to recover from water stress. Those stressed in PEG-6000 were transferred to a Hoagland solution without PEG-6000. Most stressed plants were rewatered at 25°C although some plants were watered at 5° and 10°C (Appendix Tables 3 and 4) in 2 experiments.

Preparation of the Enzyme and Enzyme Assay. The same procedure was employed in the preparation and assay of nitrate reductase as previously reported (8), except that all the tissue in this study was homogenized with Polyclar AT (1g/g tissue). Leaf tissue was sampled both when the plants were under stress and at different stages during recovery from the water stress.

Nitrate and Protein Analysis. Nitrate in the enzyme extract was determined using an Orion Research Ionalyzer, Specific Ion Meter, Model 401. The biuret method was used to determine the amount of protein using bovine serum albumin as standard (24).

Growth Measurement. The elongation of the 4th leaf was measured from the base of the blade of the third leaf to the tip of the fourth leaf during the development of water stress and during recovery from water stress. The fourth leaf was selected because being next to the youngest

leaf it was the most rapidly expanding and was coming out prominently at the base of the third leaf, which was used as the starting point when the length of the leaf was measured.

Cycloheximide Application. Cycloheximide, (3-[2-(3, 5-Dimethyl-2-oxo-cyclohexyl) - 2 hydroxyethyl]-glutarimide) was applied by spraying to runoff a 6.6×10^{-7}M solution which contained Tween 20 (2 drops/150 ml solution). This concentration was higher than that reported to inhibit protein synthesis (9, 19, 23, 26, 30). The control plants were similarly sprayed with tap water plus Tween 20. The seedlings were rewatered carefully at the base to prevent the cycloheximide from being washed off the seedlings.

Post-stress Induction of Nitrate Reductase. Plants were grown in the absence of nitrate by replacing the KNO_3 and $Ca(NO_3)_2$ salts of the Hoagland solution with equimolar concentrations of the 3 nutrients with $(NH_4)_2SO_4.7H_2O$, $CaCl_2.H_2O$ and KCl to obtain a nitrate-free solution. The seedlings were grown in this modified Hoagland solution until the early 5-leaf stage. Water stress was applied by using PEG-6000. Induction of the enzyme was initiated by supplying the seedlings with 1/3 strength Hoagland solution which contained nitrate but no PEG-6000.

RESULTS

Leaf Elongation. The elongation of corn leaves was previously (8) shown to be markedly decreased by a slight water stress; growth almost stopped at the relatively low water deficit of 6.5%. This growth inhibition can be quickly reversed. Severely stressed corn seedlings (in which growth had stopped) apparently renewed growth within 4 hr after the plants were supplied with water (Table 1 and Appendix Table 5). There was an apparent surge of growth in the early part of the recovery period; the growth measured in the first 4 hr accounted for approximately 75% of the growth made by the plant during the first 12 hr after rewatering. This sudden surge of growth was followed by a slower rate of elongation for the next 8 hr, but the total amount of growth of the recovered plants was about the same as the non-stressed plants over the entire 96 hr experimental period.

Post-stress Nitrate Reductase. The nitrate reductase activity in corn plants recovering from water stress under continuous light is reported in Table 2. The plants were usually turgid within 2 hr and were completely recovered from water deficit within 4 hr after rewatering (Table 1). However, enzyme activity did not increase significantly until 18 hr after rewatering (Table 2) and in some experiments more than 24 hr were required for a significant increase in activity.

Table 3 shows the nitrate reductase activities of control and water-stressed plants before and after recovery under continuous darkness. Enzyme activity in stressed plants failed to recover in darkness (Table 3 and Appendix Table 6). In fact, the activity in the control tissue declined to the level of the stressed tissue after 24 hr, and both control and stressed tissue declined further during subsequent 24 hr.

It was of interest to establish whether the increase in nitrate reductase activity in the stressed and rewatered plants under illumination resulted from synthesis of new enzyme or reactivation of preformed enzyme. Cycloheximide, an inhibitor of cytoplasmic protein synthesis (19), was employed to test these possibilities. The rationale of the experiment was that any activity found in the cycloheximide-treated plants should result from enzyme that was formed before cycloheximide treatment. It should be noted that the plants that were treated with cycloheximide became slightly chlorotic and showed some signs of wilting similar to that observed in slightly water-stressed plants. Plants recovering from stress in continuous light and in the absence of cycloheximide exhibited a striking recovery of enzyme activity within 12 hr (Table 4 and Appendix Table 7). The presence of cycloheximide did not prevent this recovery of activity, but did appear to reduce it. The nitrate reductase activity fell in both the cycloheximide-treated

control and stressed plants 36 and 48 hr after treatment.

In a number of experiments (e.g., Table 4) an apparent diurnal fluctuation of nitrate reductase activity was observed. The data in Table 4 suggest that 6.6×10^{-7}M cycloheximide, a concentration sufficient to completely inhibit cytoplasmic protein synthesis (9,23, 26), reduced but did not completely block this cyclical fluctuation. In another experiment, however, the same concentration of cycloheximide did eliminate this fluctuation in the controls but not in the stressed and rewatered plants (cf Appendix Table 7).

Induction of Nitrate Reductase after Stress. Since nitrate reductase is an enzyme induced by the presence of nitrate (10), experiments were conducted to determine if water stress can influence subsequent induction. Seedlings were grown and stressed in the absence of nitrate, and nitrate was supplied during the recovery period. Before addition of nitrate, some nitrate was present in the tissue as well as some reductase activity (Table 5). Upon addition of nitrate, seedlings grown in continuous light rapidly accumulated nitrate and nitrate reductase activity reapidly increased. Enzyme activity became higher in stressed than in the control seedlings, and again the diurnal fluctuation of activity was distinct (Table 5 and Appendix Tables 8 and 9). There was no relationship between nitrate content and the cyclical enzyme activity.

When seedlings recovered from water stress in continuous darkness, enzyme activity increased following addition of nitrate regardless of prior water stress (Table 6 and Appendix Table 10). In darkness, however, enzyme activity was equal in both control and stressed plants, and it declined after 12 hr. The seedlings accumulated considerably less nitrate in the dark than in light.

DISCUSSION

Recovery of Growth. Our observation of a rapid response of growth to release from water stress agrees with the findings of Hsiao et al. (18) and Raschke (27). In tomatoes, this faster growth of the stressed plants after rewatering has also been reported (12, 13). In corn, this growth after rewatering was claimed by Acevedo et al. (1) to be merely a resumption of a postponed event. Our results (Table 1 and Appendix Table 5) show a sudden burst of growth during the first hr of recovery period followed by a growth rate slower than the control plants during the 4 to 12 hr period after rewatering. There is no obvious physiological explanation for this initial high growth rate in the stressed and rewatered plants. It is likely that the desiccated tissue had actually shrunken due to loss of water, and that the initial burst of growth was a consequence of the re-entry of water making the cells turgid and thus causing the tissue to expand to its initial length. This could happen with little actual growth occurring, as illustrated by the slower growth rate during the 4 to 12 hr period after rewatering (Table 1 and Appendix Table 5).

Recovery of Nitrate Reductase. Water stress was overcome within 4 hr after rewatering (Table 1). However, the growth rates of the formerly stressed plants were unlike the control plants in both the 0 to 4 and 4 to 12 hr

periods following rewatering. Growth rates of the two groups of plants were alike in the 12 to 24 hr period. Presumably, during the first two periods the protein synthesizing systems were being regenerated so that the protein required for growth could be made anew. This may account for the observed delay in the recovery of nitrate reductase activity in previously stressed plants. It should be noted that in some experiments (Appendix Tables 3 and 4), there was a substantial increase in nitrate reductase activity in the stressed plants 6 hr after rewatering in the light, suggesting that the protein synthesizing system was being regenerated 6 hr after the plants were rewatered. Of course, the possibility that water stress did not completely inactivate nitrate reductase activity can not be overlooked, as suggested by our data from the cycloheximide experiment (Table 4). According to Beevers *et al.* (4), light increases nitrate reductase activity indirectly by increasing the availability within the cells of the nitrate needed for its induction. The light dependence of nitrate uptake has been reported by Chen and Reis (7) to be saturated at approximately 300 ft-c. The quality, duration and intensity of light has been studied in wheat by Harper and Paulsen (15), who found that nitrate reductase activity was greater under blue light and long photoperiods and that the light effects on nitrate reductase were all highly dependent on the

concentration of nitrate in the growing medium. In the present study, the concentration of nitrate in the growing medium probably was not a factor since both the control and the stressed plants were supplied with the same amount of nitrate.

The lag in recovery of enzyme activity in comparison to growth may be related to the findings of Hsiao (17) that water stress caused a shift of ribosomes from polymeric to the monomeric form starting 30 min after the initiation of stress. The shift was reversed when the plants were re-watered but the reversal always followed the change in growth and did not precede it.

The cycloheximide experiments in this study were performed to find out if the synthesis of new enzyme were solely responsible for the observed recovery of nitrate reductase activity upon release from stress. If this were so, then stressed plants treated with cycloheximide should not have exhibited recovery of activity upon rewatering. The increase of nitrate reductase activity in the rewatered stressed plants in the presence of cycloheximide in our study suggests that reactivation of pre-existing enzyme was a factor in the recovery. Losada *et al.* (25) have reported a similar reactivation of an NH_4^+-inhibited enzyme. The greater nitrate reductase activity observed in the recovered plants that were not treated with cycloheximide following removal of water stress could therefore be the summation

of both enzyme reactivation and synthesis.

In the dark, there was a steady loss of nitrate reductase activity in the control tissue as observed by others (14, 20). In contrast, the activity in the rewatered, stressed plants, although initially only 1/3 that of the controls, remained essentially constant for the first 24 hr in darkness. It is not known what causes the loss of nitrate reductase activity. It could be that it is inherently an unstable molecule which spontaneously becomes inactive. Or, alternatively, some compounds, possibly reaction products, may function in its inactivation. Ammonia, various amino acids, and even a specific protein have all been suggested for this role (10, 22, 25). It is interesting to speculate that perhaps the apparent stability of nitrate reductase in the rewatered, stressed plants was due to a low level of these postulated inactivating factors.

Induction of Nitrate Reductase. Many different factors have been found to induce nitrate reductase in different plants or organisms. Some of these are light (4, 7, 28), 0_2 and $C0_2$ (21, 28), and molybdenum (2). The factor which is the most commonly used inducer of the enzyme is nitrate (10). Nitrate has been reported in a wide variety of plants to induce nitrate reductase (5). However, apparently not all of the nitrate which accumulates in the plant system functions in the induction of

nitrate reductase. The enzyme begins to develop immediately, even before any accumulation of nitrate occurs, and very little nitrate is required to induce the enzyme (11). On the other hand, it was reported by Filner (11) that cells with 10 times more nitrate than the external concentration began to lose enzyme activity when shifted to a nitrate-free medium even if only a small per cent of the internal nitrate had been consumed. These above observations suggest that only a small portion of the nitrate in the cells induces the enzyme and that the portion which induces or maintains the enzyme has a very short half-life.

The induced nitrate reductase activity of the rewatered stressed plants in the light (Table 5), which showed twice the activity of the control, suggests that the postulated inactivating factors (e.g., ammonia, amino acids, reaction products) were reduced or missing in the tissue that recovered from water stress.

Nitrate reductase activity was induced in both the control and stressed plants in the dark (Table 6 and Appendix Table 10). Nitrate reductase has been induced in the dark by other workers (4, 29) by supplying nitrate. Hordeum vulgare, which had not previously been exposed to nitrate, was induced to form nitrate reductase in the dark when nitrate was supplied at the beginning of the dark period (31). The nitrate reductase activity in this plant

also increased in the dark until the effect of the previous light period was exhausted. The increase in nitrate reductase activity 12 hr after supplying NO_3^- in the dark observed in the present induction study (Table 6) also suggests some carry-over of the light effect into the dark. It may be pointed out here that nitrate reductase activity of ammonia-grown barley also increased after a 9-hr lag period when supplied with NO_3^- in the dark and then decreased to nearly the original level between 12 and 24 hr (31). This is consistent with our observation in corn in the present study.

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Table 1. Leaf elongation of corn seedlings during recovery from water stress under continuous light.

The plants were stressed for 48 hr in vermiculite. Leaf elongation and water deficit were measured during the last 24 hr of stress and at different time periods after rewatering. The data are averages of 3 replications.

Time Period	Increase in Length	
	Control	Stressed
hr	mm/hr	
Before Recovery		
24-48	0.77 ± 0.02 (2.2)[1]	0.00 ± 0.00 (36.2)
During Recovery		
0-4	0.75 ± 0.14 (2.1)	1.75 ± 0.11 (3.4)
4-12	0.75 ± 0.04 (2.5)	0.41 ± 0.06 (2.8)
12-24	0.83 ± 0.06 (2.3)	0.76 ± 0.06 (2.2)
24-48	0.76 ± 0.02 (3.0)	0.76 ± 0.04 (3.8)
48-72	0.70 ± 0.02 (1.8)	0.73 ± 0.02 (2.5)
72-96	0.63 ± 0.02 (1.5)	0.65 ± 0.05 (1.3)

[1]Numbers in parentheses are water stress measurements in % water deficit.

Table 2. Nitrate reductase activity in corn seedling leaves during recovery from water stress under continuous light.

Water deficit and nitrate reductase activity of plants stressed for 48 hr in vermiculite were determined. The plants were rewatered and their nitrate reductase activity and water deficit were again measured at different periods during recovery. The data are averages of 3 replications.

Recovery Period	Control	Stressed
hr	umoles NO_2^-/mg protein·min	
0	1.20 ± 0.10 (1.6)[1]	0.61 ± 0.05 (20.2)
6	1.18 ± 0.09 (2.7)	0.61 ± 0.06 (3.4)
12	1.20 ± 0.08 (2.9)	0.73 ± 0.10 (3.1)
18	1.16 ± 0.10 (3.2)	1.25 ± 0.10 (3.0)

[1]Numbers in parentheses are water stress measurements in % water deficit.

Table 3. Nitrate reductase activity in corn seedling leaves during recovery from water stress under continuous darkness.

Procedure as in Table 2. The data are averages of 3 replications.

Recovery Period	Control	Stressed
hr	umoles NO_2^-/mg protein.min	
0	1.94 ± 0.03 (2.0)[1]	0.57 ± 0.04 (32.0)
6	1.43 ± 0.02 (1.3)	0.49 ± 0.04 (3.0)
12	1.11 ± 0.04 (1.3)	0.54 ± 0.02 (1.4)
18	0.72 ± 0.03 (1.4)	0.45 ± 0.06 (1.8)
24	0.56 ± 0.04 (1.6)	0.83 ± 0.03 (1.5)
48	0.21 ± 0.02 (2.2)	0.18 ± 0.01 (2.4)

[1]Numbers in parentheses are water stress measurements in % water deficit.

Table 4. Effect of cycloheximide (CH) on nitrate reductase activity in corn seedling leaves recovering from water stress under continuous light.

Nitrate reductase activity and water deficit were determined and the plants drenched with 6.6×10^{-7} CH solution 48 hr after stress in vermiculite. Water deficit and nitrate reductase activity were also measured every 12 hr after CH application and rewatering. The data are averages of 3 replications.

Recovery Period	Control	Control + CH	Stressed	Stressed + CH
hr	umoles NO_2^-/mg protein·min			
0	1.14 ± 0.12(1.8)[1]	0.98 ± 0.14(1.4)	0.25 ± 0.02(16.2)	0.26 ± 0.04(18.2)
12	0.44 ± 0.12(1.8)	0.16 ± 0.05(1.8)	0.61 ± 0.19(2.8)	0.44 ± 0.02(1.8)
24	1.34 ± 0.13 (2.3)	0.80 ± 0.02(1.7)	2.30 ± 0.08(2.2)	1.24 ± 0.28(2.2)
36	0.74 ± 0.14(2.8)	0.07 ± 0.02(2.5)	0.44 ± 0.06(3.7)	0.12 ± 0.03(3.5)
48	1.12 ± 0.02(2.9)	0.28 ± 0.02(2.7)	1.64 ± 0.08(3.5)	0.40 ± 0.02(3.5)

[1]Numbers in parentheses are water stress measurements in % water deficit.

Table 5. Induction of nitrate reductase activity in corn seedling leaves during recovery from water stress under continuous light.

Water deficit, nitrate reductase activity and NO_3^- concentration were determined 48 hr after stress in PEG-6000 and at intervals after the plants were removed from PEG-6000. NO_3^- was supplied immediately after the 0-hr measurements. The data are averages of 3 replications.

Recovery Period	Nitrate Reductase Activity		NO_3^- Concentration	
	Control	Stressed	Control	Stressed
hr	umoles NO_2^-/mg protein·min		mg/g dry wt	
0	0.20 ±0.02(2.5)[1]	0.20 ±0.06(22.5)	10.97 ± 0.42	9.45 ± 1.45
6	0.46 ±0.06(1.9)	0.36 ±0.03(1.9)	28.42 ± 1.46	22.08 ± 1.41
12	1.06 ±0.06(2.6)	2.40 ±0.10(5.2)	47.40 ± 1.36	48.90 ± 4.57
24	0.92 ±0.10(3.7)	1.42 ±0.16(3.8)	31.08 ± 4.69	42.88 ± 2.55
36	1.12 ±0.15(3.3)	2.31 ±0.10(2.9)	90.15 ±13.05	37.95 ± 2.50

[1]Numbers in parentheses are water stress measurements in % water deficit.

Table 6. Induction of nitrate reductase activity in corn seedling leaves during recovery from water stress under continuous darkness.

Procedure as in Table 5. The data are averages of 3 replications.

Recovery Period	Nitrate Reductase Activity		NO_3^- Concentration	
	Control	Stressed	Control	Stressed
hr	umoles NO_2^-/mg protein·min		mg/g dry wt	
0	0.17 ±0.02(1.9)[1]	0.14 ±0.03(21.2)	7.20 ± 1.80	6.90 ± 2.0
6	0.33 ±0.03(2.1)	0.29 ±0.00(3.6)	10.00 ± 0.97	13.98 ± 4.78
12	1.27 ±0.03(2.5)	1.24 ±0.06(2.4)	13.28 ± 0.78	15.00 ± 0.57
24	0.63 ±0.04(1.0)	0.52 ±0.04(1.4)	12.82 ± 0.77	14.65 ± 0.39
36	0.12 ±0.02(1.7)	0.10 ±0.01(1.8)	23.28 ± 2.20	25.10 ± 0.71

[1]Numbers in parentheses are water stress measurements in % water deficit.

A Short Paper on

THE EFFECT OF TEMPERATURE ON NITRATE REDUCTASE ACTIVITY OF CORN SEEDLINGS[1]

Rafael P. Creencia

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[1]Part of a dissertation submitted by the author in partial fulfillment of the requirement for a Ph.D. in Plant Soil Sciences at the University of Massachusetts.

ABSTRACT

Nitrate reductase activity of corn (Zea mays, L., cv. WF9 x M14, Crow's hybrid corn, Milford, Ill.) was determined under temperatures of 15°, 25°, 35° and 43°C. Under continuous light activity decreased with time at each temperature but the rate of decrease was slower at 25° than at the lower or higher temperatures. Under continuous light, loss in activity was not observed at 25°C in seedlings previously grown at either 15° or 35°C. Under continuous darkness, the rate of activity loss was related to increasing temperatures. The activity was highest at 15°C in plants kept on a 12-hr light and 12-hr dark cycle. The role of inactivating factors, which may be increasing at higher temperatures, was discussed.

INTRODUCTION

A preliminary experiment (Appendix Table 11) showed that nitrate reductase activity was lower at 35°C than at 21.1°C. Since water stress and high temperature frequently occur together in nature, it has been difficult to assess the independent effects of the two factors in plants. Plant metabolism is highly dependent on these two overlapping factors. While it is true that most metabolic reactions take place in a water milieu (13), it is equally true that these reactions can be greatly influenced by temperature.

This study is concerned with the effect of temperature *per se* on nitrate reductase activity apart from the effect of water stress.

MATERIALS AND METHODS

Seeds of Zea mays, L. (cv. WF9 x M14, Cro'w hybrid corn, Milford, Ill.) were soaked in aerated tap water for 24 hr and sown in vermiculite. The plants were supplied with full strength Hoagland (4) solution at least twice a week. The seedlings when at the 3 to 4 cm stage were removed from the vermiculite and placed in holes on circular styrofoam boards. These boards, each holding 16 seedlings, were placed on 1 liter black plastic pots filled with 1/3 strength, aerated Hoagland solution, which was changed twice a week. Seedlings were grown in the greenhouse until they reached the early 5-leaf stage, at which time they were transferred into a growth chamber where the light, temperature and relative humidity were controlled. The light intensity was 3,300 ft-c, and the relatively humidity was kept as close to 75% as possible to prevent water stress. The plants were kept at 25°C under 12-hr light and 12-hr dark periods for 2 days to allow them to adjust to growth chamber condition before each of the temperature treatments was started.

Temperatures of 15°, 25°, 35° and 43°C were used under continuous light, continuous darkness or 12-hr light and 12-hr dark cycle. Nitrate reductase activity was determined at different time intervals under these

temperatures and light conditions.

In other experiments, seedlings were kept for 2 days at 15° or 35°C after which the temperatures were shifted to 25°C. Nitrate reductase activity was determined before and after a temperature shift was made.

Measurement of water deficit, preparation of the enzyme and enzyme assay and protein analysis were made following the same procedures described in the previous papers (1, 2).

RESULTS

In spite of the continuously available water supply from the nutrient solution, the water deficit in corn plants that were held at 43°C under continuous light rose to more than 5% (Table 1). This water deficit was observed 6 hr after the imposition of the temperature treatments and stayed at this level to the end of the 36-hr experimental period. On the other hand, the water deficit remained below 5% in plants that were under 15°, 25° and 35°C. The initial nitrate reductase activities were different at the different temperatures due probably to the differences in the batches of seedlings used for each temperature treatment. The enzyme activities at the different temperature levels decreased with time under continuous light. The decrease at 35° and 43°C appears much more rapid than at 25°C. At 15°C the rate of decrease was intermediate between 25° and the higher temperatures. The concentration of CO_2 in the growth chamber was measured to find out if the decline in the nitrate reductase activity at different temperatures could be explained by a condition of limiting CO_2. Results (Appendix Table 12) showed that CO_2 was always higher than that normally present in ambient air. The possibility of ethylene accumulation in the growth chamber air was also examined (Appendix Figure 1) but this could not be detected in the growth chamber atmosphere.

A striking effect of temperature on nitrate reductase activity was observed when the plants were kept under continuous darkness (Table 2). Activity was lost, and the loss was greater at higher than at lower temperatures. More than 50% of the initial enzyme activity of plants held at 43°C was lost within 6 hr. A similar loss of activity was observed after 12 hr at 25°C and 35°C. The loss of activity was much more gradual at 15°C than at the other 3 temperatures. Plants under 43°C collapsed after 36 hr under the dark period. The average nitrate reductase activity within the 36 hr dark period clearly showed that increasing the temperature during darkness increased the loss of enzyme activity (Table 2).

Table 3 shows the nitrate reductase activity of plants held at different temperatures under 12 hr light and 12 hr dark regime. Activities measured at the end of the light period were higher than those measured at the end of the dark period at all temperatures. Other than this variation between light and dark periods, the enzyme activities tended to keep the initial levels for the 48 hr period, except for a rather marked reduction at 43°C.

In an earlier experiment (Table 1), the nitrate reductase activities in plants that were held at 15° and 35°C decreased to a much lower level than at 25° by the end of 36 hr. It was thought that if these plants with

such low level of activity were transferred to the apparently favorable temperature (25°C), this activity might recover. The data in Table 4 show that exposure to 15° and 35° failed to result in the low level of activity shown in Table 1. Transferring the plant to 25°C resulted in the continuation of essentially the original level of activity.

DISCUSSION

High temperatures have been reported to inactivate nitrate reductase (7, 8, 9, 15). In our study, this inactivation was observed to become progressively greater with longer times at a high temperature of 43° than at the 3 lower temperatures. Water deficit in plants under this high temperature was slightly higher than in plants under 35°, 25° or 15°C. This water deficit (5%), however, is below what we previously showed (1) to hardly affect nitrate reductase activity. Onwueme *et al*. (8) also detected a slight water stress during heat exposure of barley seedlings which resulted in only a small reduction in nitrate reductase activity. Probably this small heat-induced water deficit contributed little if any to the nitrate reductase inactivation observed during the heat stress. Heat stress, therefore, must have caused loss of nitrate reductase activity of plants exposed to high temperatures. Perhaps high temperature speeded up the degradation of some enzymes including nitrate reductase. High temperature possibly increased the catabolic reactions in the plant cells which led to the formation of products which may be inhibitory to nitrate reductase. One of the heat-induced catabolic products reported was ammonia (5), which was shown to inactivate nitrate reductase in *Chlamydomonas* (6), in rice (10) and in barley (12).

The faster loss of nitrate reductase activity in the dark at higher temperature is probably due to an increased action of an inactivating system in the plant tissue. An inactivating system has been reported in barley (14) which was shown to require protein synthesis and to be inhibited by low temperature. The higher nitrate reductase activity in the light observed in plants kept under 12-hr light and 12-hr dark regime is typical of nitrate reductase (3, 11). Perhaps under this condition the low temperature of 15°C resulted in a slower degradation of the enzyme.

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Table 1. Nitrate reductase activity in leaves of water culture-grown corn seedlings at different temp under continuous light.

The temperature in the growth chamber was kept at 25°C under a 12-hr light and 12-hr dark regime for 2 days before the initial activity of the enzyme was measured. The light was made continuous and the temp was adjusted to the desired level after measuring the initial activity of the enzyme. The data under each temp were from different sets of experiments and are from averages of 3 replications.

Time Period	Temp			
	15°C	25°C	35°C	43°C
hr	umoles NO_2^-/mg protein·min			
0	2.12 ±0.11(1.0)[1]	1.50 ±0.11(1.5)	2.06 ±0.06(1.2)	1.68 ±0.21(2.7)
6	2.54 ±0.30(2.9)	1.58 ±0.17(1.8)	1.14 ±0.11(3.2)	1.15 ±0.14(5.2)
12	0.79 ±0.07(4.3)	1.49 ±0.06(2.2)	0.96 ±0.00(4.1)	0.72 ±0.05(5.1)
24	0.91 ±0.15(1,8)	1.28 ±0.17(2.0)	0.95 ±0.12(3.4)	0.64 ±0.07(5.2)
36	0.63 ±0.06(2.1)	0.96 ±0.09(2.2)	0.81 ±0.22(4.0)	0.56 ±0.04(5.2)
Average[2]	1.22	1.32	0.97	0.77

[1]Numbers in parentheses are water stress measurements in % water deficit.

[2]Average of the activities determined at different periods at designated temp excluding the initial activity.

Table 2. Nitrate reductase activity in leaves of water culture-grown corn seedlings at different temp under continuous darkness.

The temp in the growth chamber was kept at 25°C with a 12-hr light and 12-hr dark regime for 2 days before the initial activity of the enzyme was measured. Dark period was started and temp was adjusted to the desired level after measuring the initial activity of the enzyme. The data under each temp were from different sets of experiments and are averages of 3 replications.

Time Period	Temp			
	15°C	25°C	35°C	43°C
hr	umoles NO_2^-/mg protein·min			
0	1.80 ± 0.08	1.70 ± 0.16	1.81 ± 0.08	1.60 ± 0.04
6	1.27 ± 0.09	1.32 ± 0.10	1.38 ± 0.06	0.71 ± 0.03
12	1.03 ± 0.09	0.54 ± 0.10	0.59 ± 0.02	0.43 ± 0.01
24	1.19 ± 0.06	0.53 ± 0.01	0.27 ± 0.07	0.44 ± 0.05
36	0.83 ± 0.08	0.20 ± 0.02	0.08 ± 0.01	-
Average[1]	1.08	0.65	0.58	0.52

[1]Average of activities determined at different periods at designated temp excluding the initial activity.

Table 3. Nitrate reductase activity in leaves of water culture-grown corn seedlings under a 12-hr dark and 12-hr light regime.

The temp in the growth chamber was kept at 25°C under 12-hr light and 12-hr dark periods for 2 days before the initial activity of the enzyme was measured. The light and dark periods were maintained while the temp were adjusted to the desired levesl after measuring the initial activity of the enzyme. The data under each temp were from different sets of experiments and are the averages of 3 replications.

Time Period	Temp			
	15°C	25°C	35°C	43°C
hr	umoles NO_2^-/mg protein·min			
0	1.40 ± 0.04	1.52 ± 0.10	1.36 ± 0.01	1.58 ± 0.04
12 (Dark)	0.96 ± 0.02	0.78 ± 0.04	0.63 ± 0.01	0.67 ± 0.06
24(Light)	1.53 ± 0.05	1.41 ± 0.03	1.23 ± 0.04	0.80 ± 0.11
36 (Dark)	1.23 ± 0.12	0.76 ± 0.04	0.29 ± 0.04	0.73 ± 0.15
48(Light)	2.01 ± 0.19	1.41 ± 0.05	1.43 ± 0.05	1.05 ± 0.12
Average[1]	1.44	1.09	0.90	0.81

[1]Average of activities determined at different periods at the designated temp excluding the initial activity.

Table 4. Nitrate reductase activity in leaves of water culture-grown corn seedlings after shifting the temp from 15°C and from 35°C to 25°C under continuous light.

The temp was set at either 15°C or 35°C when the seedlings were transferred into the growth chamber. The initial activity was measured and the temp was shifted to 25°C after 2 days. The data under each temperature shifts were from different sets of experiment and are averages of 3 replications.

Time Period	Temp Shift	
	15°C to 25°C	35°C to 25°C
hr	umoles NO_2^-/mg protein·min	
0	1.39 ± 0.10	1.44 ± 0.09
12	1.29 ± 0.08	1.64 ± 0.13
24	1.45 ± 0.03	1.66 ± 0.03
36	1.40 ± 0.17	1.57 ± 0.16
48	1.46 ± 0.12	1.38 ± 0.10

APPENDIX

Supplementary Data

INTRODUCTORY NOTES ABOUT THE SUPPLEMENTARY DATA INCLUDED IN THE APPENDIX

1. The computer program used to fit a line of relationship between leaf water deficit and leaf water potential was included in Appendix Tables 1 and 2 for future reference. This program can be useful for any other pair of variables that need to be correlated.
2. Appendix Tables 3 and 4. Water-stressed corn seedlings were made to recover at low temperatures of 5° and 10°C. These experiments were discontinued because the growth chamber facility was inadequate to pursue this investigation. It is interesting to note that recovery in water deficit was considerably delayed at 10° and 5°C compared to those recovering at 25°C.
3. Appendix Table 5. A preliminary experiment on growth recovery after stress. The experiment was repeated, leaf elongation was measured for a longer period after rewatering and the results were included in the paper entitled "Growth and nitrate reductase activity of corn seedlings recovering from water stress.

4. Appendix Table 6 is a preliminary experiment on nitrate reductase recovery in darkness of corn seedlings after water stress. The same experiment was conducted where nitrate reductase activity was measured at more frequent intervals and presented in Table 3 of the second paper of this dissertation.
5. Appendix Table 7 is essentially the same experiment as the one presented in Table 4 of the second paper of this dissertation except that the plants in the latter were less severely stressed and thus the data on recovery are probably more meaningful.
6. Appendix Tables 8 and 9 are supplementary to the data presented in Table 5 of the second paper of this dissertation on the induction of nitrate reductase activity in the light after the corn plants were rewatered.
7. Appendix Table 10 supplements the data presented in Table 6 of the second paper in this dissertation on the induction of nitrate reductase activity in darkness after the corn plants were rewatered.
8. Appendix Table 11. The data in this table are results of a preliminary experiment on the effects of temperature on nitrate reductase activity. More experiments were conducted and the results are presented in the Tables included in the short paper on the effect of temperature on nitrate reductase activity

of corn seedlings.

9. Appendix Table 12. The CO_2 concentration in the growth chamber was measured to find out if the decline in nitrate reductase activity at different temperatures presented in Table 1 of the paper on the effect of temperature on nitrate reductase activity of corn seedlings could be explained by a condition of limiting CO_2. Results showed that CO_2 was always higher than that normally present in ambient air.

10. Appendix Figure 1. The possibility of ethylene accumulation in the growth chamber air was also examined using a gas chromatograph. The figure show that ethylene could not be detected in the growth chamber atmosphere. The data in Appendix Figure 1 show that the chromatograph used could not detect ethylene concentrations below 1 ppm. It is not known whether ethylene concentration at less than 1 ppm can inhibit nitrate reductase activity. This question has not been clarified.

Appendix Table 1. Program used to determine the best fit line relationship between water deficit and leaf water potential in corn seedlings.

```
LIST
100 PROGRAM LSWF
101*LEAST SQUARE FIT FOR PAENG AND MAMENG
160 DIMENSION X(50),Y(50),A(50,50),B(50),C(50),P(50)
161  INPUT ,M
162 READ ,NUMBER
164 DO 1  I=1,NUMBER
166 READ ,X(I),Y(I)
168†1 CONTINUE
170 MX2=M*2
180 DO 13  I=1,MX2
190 P(I)=0.0
200 DO 13  J=1,NUMBER
210†13 P(I)=P(I)+X(J)**I
220 N=M+1
230 DO 30  I=1,N
240 DO 30 J=1,M
250 K=I+J-2
260 IF(K)29,29,28
270†28 A(I,J)=P(K)
280 GO TO 30
290†29 A(I,I)=NUMBER
300†30 CONTINUE
310 B(1)=0.0
320 DO 21  J=1,NUMBER
330†21 B(1)=B(1)+Y(J)
340 DO 22  I=2,N
350 B(I)=0.0
360 DO 22  J=1,NUMBER
370†22 B(I)=B(I)+Y(J)*X(J)**(I-1)
380 NM1=N-1
390 DO 60 K=1,NM1
400 KP1=K+1
410 L=K
420 DO 40 I=KP1,N
430 IF(ABSF(A(I,K))-ABSF(A(L,K)))40,40,41
440†41 L=I
450†40 CONTINUE
460 IF(L-K)50,50,45
```

Appendix Table 2. Continuation of the program used to determine the best fit line relationship between water deficit and leaf water potential in corn seedlings.

```
470 45 DO 46 J=K,N
480 TEMP=A(K,J)
490 A(K,J)=A(L,J)
500 46 A(L,J)=TEMP
510 TEMP=B(K)
520 B(K)=B(L)
530 B(L)=TEMP
540 50 DO 60 I=KP1,N
550 FACTOR=A(I,K)/A(K,K)
560 A(I,K)=0.0
570 DO 61 J=KP1,N
580 61 A(I,J)=A(I,J)-FACTOR*A(K,J)
590 60 B(I)=B(I)-FACTOR*B(K)
600 C(N)=B(N)/A(N,N)
610 I=NM1
620 71 IP1=I+1
630 SUM=0.0
640 DO 70 J=IP1,N
650 70 SUM=SUM+A(I,J)*C(J)
660 C(I)=(B(I)-SUM)/A(I,I)
670 I=I-1
680 IF(I)80,80,71
688 80 CONTINUE
690 XX=0.
691 PRINT 1001
699 ITER=11
700 DO 2 IREP=1,ITER
701 XREP=IREP-1
702 XX=XREP*5.
720 SC=0.0
730 DO 25 JJ=1,M
740 I=M+1-JJ
750 SC=SC*XX+C(I)
760 25 CONTINUE
761 2 PRINT 1000,XX,SC
1000 FORMAT (2F10.3)
1001 FORMAT (7X*WD*7X*BARS*)
1500 END
1999 ENDPROG
2000 ##
```

Appendix Table 3. Nitrate reductase activity in corn seedling leaves during recovery from water stress at different temp and under continuous light.

Water deficit and nitrate reductase activity of plants stressed for 48 hr in vermiculite were determined. The plants were rewatered and their nitrate reductase activity and water deficit were again measured at different periods during recovery. The data are averages of 3 replications.

Recovery Period	Control		Stressed	
	25°C	5°C	25°C	5°C
hr	umoles NO_2^-/mg protein·min			
0	1.22 (1.9)[1]	1.39 (2.5)	0.57 (35.8)	0.71 (29.2)
6	0.72 (2.2)	1.12 (21.4)	0.79 (3.1)	0.72 (20.3)
12	1.26 (o.0)	0.37 (26.8)	0.35 (1.9)	0.32 (18.4)

[1]Numbers in parentheses are water stress measurements in % water deficit.

Appendix Table 4. Nitrate reductase activity in corn seedling leaves during recovery from water stress at different temp and under continuous light.

Procedure at in Appendix Table 3. The data are averages of 3 replications.

Recovery Period	Control		Stressed	
	25°C	10°C	25°C	10°C
hr	umoles NO_2^-/mg protein·min			
0	1.16 (2.9)[1]	1.44 (3.1)	0.58 (20.6)	0.30 (17.9)
2	1.35 (2.5)	1.37 (2.8)	0.56 (4.0)	0.36 (25.8)
4	0.80 (2.4)	1.26 (2.7)	0.60 (2.3)	0.24 (31.6)
6	1.32 (2.7)	2.02 (4.3)	0.81 (2.4)	0.80 (26.2)
12	1.40 (1.9)	2.07 (2.7)	0.86 (2.9)	0.84 (8.0
24	1.32 (3.6)	1.64 (3.5)	0.50 (3.2)	0.74 (2.7)

[1]Numbers in parentheses are water stress measurements in % water deficit.

Appendix Table 5. Leaf elongation of corn seedlings during recovery from water stress under continuous light.

The plants were stressed for 48 hr in vermiculite. Leaf elongation and water deficit were measured during the last 24 hr of stress and at different time periods after rewatering. The data are averages of 3 replications.

Time Period	Increase in Length	
	Control	Stressed
hr	mm/hr	
Before Recovery		
24-48	0.75 (5.3)[1]	0.00 (31.2)
During Recovery		
0-4	0.98 (2.7)	1.65 (3.9)
4-12	0.86 (2.2)	0.48 (2.3)
12-24	0.75 (2.3)	0.81 (2.4)
24-48	0.75 (1.9)	0.75 (2.2)

[1]Numbers in parentheses are water stress measurements in % water deficit.

Appendix Table 6. Nitrate reductase activity in corn seedling leaves during recovery from water stress under continuous darkness.

Water deficit and nitrate reductase activity of plants stressed for 48 hr in vermiculite were determined. The plants were rewatered and their nitrate reductase activity and water deficit were again measured at different periods during recovery. The data are averages of 3 replications.

Recovery Period	Control	Stressed
hr	umoles NO_2^-/mg protein·min	
0	1.41 (2.2)[1]	0.42 (22.7)
24	0.72 (2.0)	0.33 (2.6)
48	0.16 (2.2)	0.09 (2.2)

[1]Numbers in parentheses are water stress measurements in % water deficit.

Appendix Table 7. Effect of cycloheximide (CH) on nitrate reductase activity in corn seedling leaves recovering from water stress under continuous light.

Nitrate reductase activity and water deficit were determined and the plants drenched with 6.6×10^{-7}M CH solution 48 hr after stress in vermiculite. Water deficit and nitrate reductase activity were also measured at intervals after CH application and rewatering. The data are average of 3 replications.

Recovery Period	Control	Control + CH	Stressed	Stressed + CH
hr		umoles NO_2^-/mg protein·min		
0	0.72 (1.9)[1]	0.80 (2.2)	0.04 (37.6)	0.04 (34.5)
6	0.91 (2.7)	0.80 (3.4)	0.15 (3.0)	0.23 (3.3)
12	0.57 (1.6)	0.48 (2.4)	0.81 (2.6)	0.06 (3.0)
24	1.73 (2.8)	0.40 (2.6)	2.10 (2.3)	1.36 (2.4)
36	0.59 (3.9)	0.25 (3.8)	0.55 (3.6)	0.24 (2.6)
48	1.44 (2.5)	0.23 (3.3)	2.05 (2.9)	0.34 (2.4)

[1]Numbers in parentheses are water stress measurements in % water deficit.

Appendix Table 8. Induction of nitrate reductase activity in corn seedling leaves during recovery from water stress under continuous light.

Water deficit and nitrate reductase activity were determined 48 hr after stress in PEG-6000 and at intervals after the plants were removed from PEG-6000. The data are averages of 3 replications.

Recovery Period	Control	Stressed
hr	umoles NO_2^-/mg protein·min	
0	0.15 (2.9)[1]	0.11 (23.0)
6	0.38 (2.7)	0.28 (2.7)
12	1.23 (2.6)	1.87 (2.7)
24	1.02 (2.6)	1.52 (2.7)
36	1.48 (2.7)	2.60 (2.8)

[1]Numbers in parentheses are water stress measurements in % water deficit.

Appendix Table 9. Induction of nitrate reductase activity in corn seedling leaves during recovery from water stress under continuous light.

Procedure as in Appendix Table 8. The data are averages of 3 replications.

Recovery Period	Control	Stressed
hr	umoles NO_2^-/mg protein·min	
0	0.17 (2.4)[1]	0.13 (25.8)
6	0.24 (2.3)	0.19 (2.2)
12	1.11 (2.2)	1.56 (2.4)
24	0.85 (2.2)	1.04 (2.5)
36	1.29 (2.1)	1.93 (2.5)

[1]Numbers in parentheses are water stress measurements in % water deficit.

Appendix Table 10. Induction of nitrate reductase activity in corn seedling leaves during recovery from water stress under continuous darkness.

Procedure as in Appendix Table 8. The data are averages of 3 replications.

Recovery Period	Control	Stressed
hr	umoles NO_2^-/mg protein·min	
0	0.16 (3.0)[1]	0.12 (17.8)
6	0.42 (2.8)	0.51 (3.1)
12	1.39 (3.0)	1.29 (2.8)
24	0.72 (2.4)	0.63 (2.8)
36	0.14 (2.9)	0.11 (2.1)

[1]Numbers in parentheses are water stress measurements in % water deficit.

Appendix Table 11. Nitrate reductase activity of soil-grown corn seedling leaves at different temp under 12 hr light and 12 hr dark regime.

The plants were grown in soil in the greenhouse and were transferred into a growth chamber at their early 5-leaf stage. The temperature in the growth chamber was adjusted to 21.1°C or 35°C after 2 days and nitrate reductase activity was determined every 24 hr. The data are averages of 3 replications.

Time Period	Temp	
	21.1°C	35°C
hr	umoles NO_2^-/mg protein·min	
24	2.38 ± 0.08	2.37 ± 0.04
48	2.22 ± 0.06	1.43 ± 0.04
72	2.23 ± 0.09	1.50 ± 0.07

Appendix Table 12. CO_2 concentration in the growth chamber at different times and at different temp under continuous light.

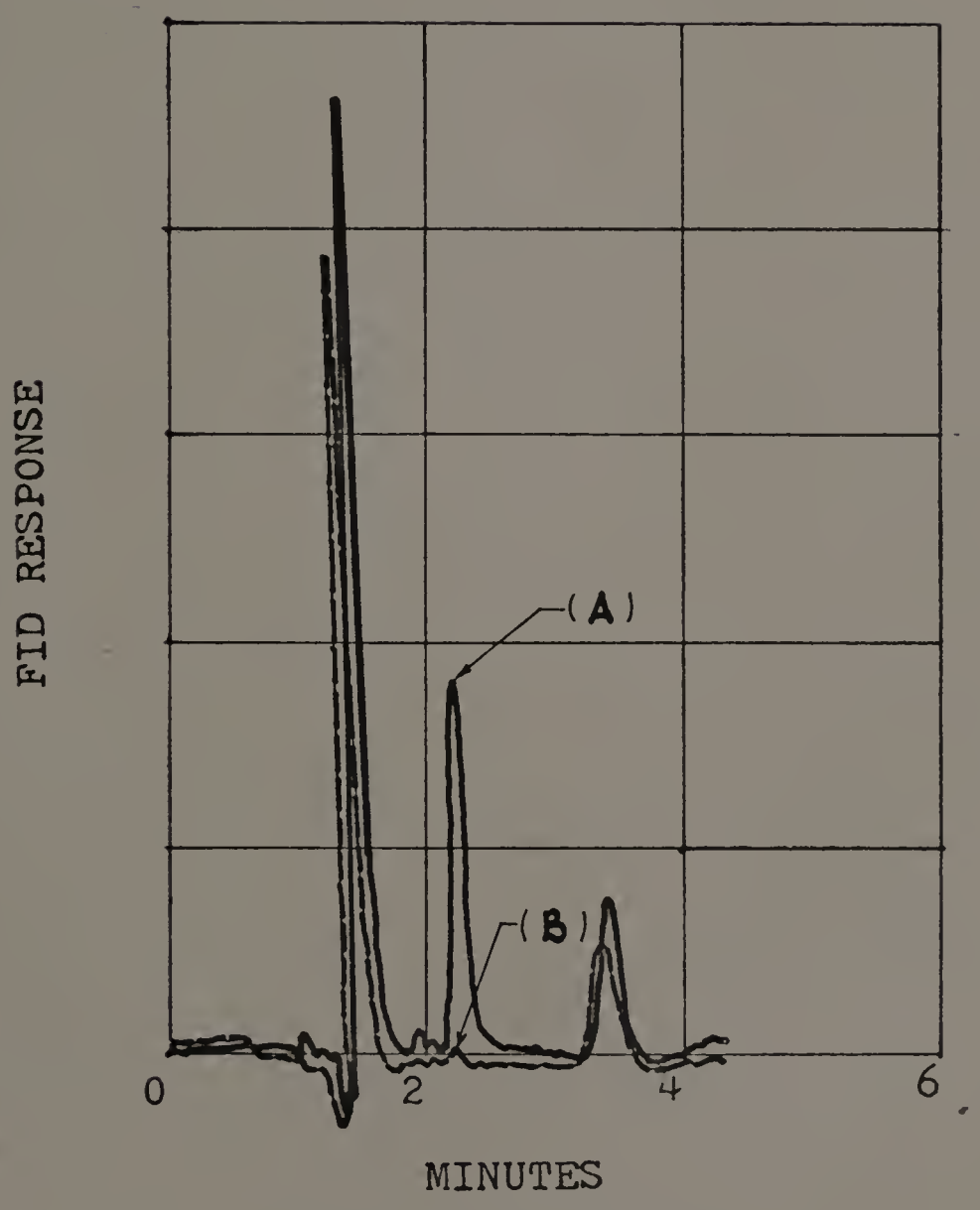
CO_2 concentration was determined at different times after the seedlings were transferred into the growth chamber using the Kitagawa CO_2 low range tubes which detect CO_2 concentrations of 100-7000 ppm. (Union Industrial Equipment Corp., Fall River, Massachusetts). The data are averages of 2 determinations.

Temp	CO_2 Concentration	
	72 Hr	84 Hr
°C	ppm	
15	1500	1300
25	1350	1050
35	900	800
43	900	800

APPENDIX FIGURE 1

Gas chromatogram showing ethylene peaks for 5 ppm standard ethylene and for growth chamber air.

Duplicate 1-ml samples of 5 ppm standard ethylene and of the air from the growth chamber where the corn seedlings were grown were analyzed with a Varian Aerograph Model 600-D gas chromatograph equipped with a flame ionization detector. A 6-ft long, 1/8 in ID stainless steel column containing 60-80 mesh activated alumina was employed at 70°C. The arrows indicate the ethylene peaks (A) for 5 ppm ethylene and (B) for the air sample.



APPENDIX FIGURE 1